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ORIGINAL ARTICLE

Strontium ranelate improves bone strength in ovariectomized rat by positively influencing bone resistance determinants

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Abstract
Summary Treatment of adult ovariectomized (OVX) rats with strontium ranelate prevented vertebral biomechanics degradation as a result of the prevention of bone loss and micro-architecture deterioration associated to an effect on intrinsic bone material quality. Strontium ranelate influenced the determinants of bone strength by prevention of ovariectomy-induced changes which contribute to explain strontium ranelate antifracture efficacy.
Introduction Strontium ranelate effects on the determinants of bone strength in OVX rats were evaluated.
Methods Adult female Sprague–Dawley rats were OVX, then treated daily for 52 weeks with 125, 250, or 625 mg strontium ranelate/kg. Bone strength, mass, micro-architecture, turnover, and intrinsic quality were assessed.
Results Strontium ranelate prevented ovariectomy-induced deterioration in mechanical properties with energy necessary for fracture completely maintained vs. SHAM at 625 mg/kg/day, which corresponds to the clinical dose. This was related to a dose-dependent effect on bone volume, higher trabeculae number, and lower trabecular separation in strontium ranelate vs. OVX. Load and energy required to induce lamella deformation were higher with strontium ranelate than in OVX and in SHAM, indicating that the bone formed with strontium ranelate is able to withstand greater damage before fracture. Bone formation was maintained high or even increased in strontium ranelate as shown by mineralizing surfaces and alkaline phosphatase while strontium ranelate led to reductions in deoxypyridinoline.
Conclusion Strontium ranelate administered at 625 mg/kg/day for 52 weeks prevented OVX-induced biomechanical properties deterioration by influencing the determinants of bone strength: it prevented bone loss and micro-architecture degradation in association with an effect on intrinsic bone quality. These beneficial effects on bone contribute to explain strontium ranelate antifracture efficacy.

Keywords Bone biomechanics · Bone intrinsic quality · Bone micro-architecture · Ovariectomized rat · Strontium ranelate

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Introduction

Postmenopausal osteoporosis is still a major public health problem. Until now, most efforts to reduce the risk and

incidence of fractures have focused on therapies that either preserve skeletal mass by inhibiting osteoclastic bone resorption or reverse bone loss by stimulating osteoblastic bone formation [1]. An ideal therapy for the reversal of bone fragility would be a single therapeutic engineered to possess both antiresorbing and bone-forming properties [2].

Strontium ranelate, composed of two stable strontium atoms and ranelic acid, represents a new paradigm in the treatment of osteoporosis [3]. In phase III studies in postmenopausal osteoporotic women, strontium ranelate (2 g/day) reduced the risk of vertebral fracture, the risk of nonvertebral fracture, and the risk of hip fracture over 3 years [4, 5]. It has been shown to be effective in reducing bone loss and/or increasing bone mass and/or bone resistance in intact mice [6], rats [7], and monkeys [8] as well as in short-term rat models of osteopenia, including ovariectomy-induced bone loss [9, 10] and immobilization [11]. Furthermore, in vitro studies have shown that strontium ranelate has an original mechanism of action acting by reducing osteoclastic bone resorption [12, 13] while at the same time stimulating osteoblastic bone formation [14].

The clinical aim of an anti-osteoporotic treatment is to decrease the risk of fracture, an event related to bone resistance which is driven by parameters including bone mass, bone size and shape, bone turnover, bone micro-architecture, and intrinsic bone tissue quality including damage accumulation [15]. Valid techniques for noninvasive assessment of bone strength in human patients are currently not available and fracture risk reduction is only an indirect marker of bone strength. Therefore, evaluation of the drug-induced effects on these variables in animals can yield valuable insights into the underlying mechanisms that influence the antifracture efficacy of a therapy. The ovariectomized (OVX) rat is a well-recognized and validated model of bone loss that closely resembles the osteoporosis observed in postmenopausal women [16–18]. Therefore, the current study was carried out to evaluate the long-term bone efficacy and safety of strontium ranelate (125, 250, and 625 mg/kg/day for 12 months) on all the determinants of bone strength in OVX adult Sprague–Dawley rats, a relevant model of osteoporosis, in order to better understand clinical antifracture efficacy of this anti-osteoporotic agent. The response to strontium ranelate was evaluated using determinations of bone strength (using biomechanical testing) and of the determinants influencing bone strength: bone mass and micro-architecture (using micro-computed tomography [μCT] and static histomorphometry), bone turnover (using dynamic histomorphometry and bone turnover markers), and intrinsic bone tissue quality (using nano-indentation).

Materials and methods

Animals and treatment

The protocol for this experiment was approved by the Institutional Animal Care and Use Committee at SkeleTech where the study was performed. Six-month-old virgin female Sprague–Dawley rats (Harlan Sprague Dawley, Indianapolis, IN, USA) were randomized to treatment groups based on body weights and ovariectomy or sham surgery was performed. Treatment was initiated on the day following ovariectomy or sham surgery. Animals were individually housed in rooms with controlled temperature and relative humidity and an alternating 12-h dark/light photoperiod. Animals were fed PMI Certified Rodent Diet 5002 containing 0.80% calcium, 0.60% phosphorus, and 2.2 IU of vitamin D_3 per gram of feed. Water was available ad libitum.

Three OVX treatment groups (SR125, SR250, and SR625; n=30 animals each) were administered strontium ranelate (S12911-2, Technologie Servier, Orléans, France) at dose levels of 125, 250, and 625 mg/kg/day. These doses were based on previous studies demonstrating that strontium ranelate was effective in preventing OVX-induced bone loss and at improving bone resistance [7, 9]. Strontium ranelate was prepared weekly as a suspension in the vehicle (0.5% carboxymethylcellulose sodium salt, medium viscosity; Spectrum Chemical, Gardena, CA, USA). The control sham-operated (SHAM) group (n=24 animals) and OVX group (n=25 animals) were administered the vehicle. All treatments were administered daily by gavage (10 mL/kg) for 52 weeks.

During treatment, body weights were obtained weekly and the animals were individually pair-fed according to the average daily food consumption of the SHAM control animals. Prior to necropsies performed on week 52, all animals received a fluorochrome-labeling regimen on days 16/15 and 5/4 prior to killing to deposit double fluorochrome labels on mineralizing surfaces (calcein green, 12 mg/kg IP). At the time of necropsy, the final body weights were recorded and the animals were sedated with ketamine/xylazine and then humanely killed while still under sedation. Following killing, the uterus was removed and weighed to verify the success of ovariectomy. The lumbar vertebrae (L2 to L5) were then excised.

Bone mechanical properties

Biomechanical tests were performed in a blinded manner on LV5 vertebral body specimens using an Instron mechanical testing machine (Instron 4465 retrofitted to 5500) interfaced to a personal computer with Merlin II machine software. For each sample, the vertebral arch,

pedicle, and cranial and caudal ends of each vertebral body were removed using a low-speed diamond saw to obtain a vertebral body specimen with two parallel surfaces and a height of approximately 4 mm (width and height of the vertebral body were measured using digital calipers). The specimens were then placed between two platens and a load was applied at a displacement rate of 6 mm/min until failure. The load–displacement curve for each test was recorded: the maximum load at failure (expressed in newtons) as well as the yield load at the transition between the elastic and plastic phases of the deformation were manually selected (changes detected on the tangent). Machine software was then used to calculate the stiffness (slope of the elastic part of the curve, expressed in newtons per millimeter) and total energy absorbed (total energy absorbed, expressed in millijoules). All calculations were according to established formulae [15].

Bone histomorphometry

Bone specimens for histomorphometry (L3) were fixed in cold, 70% ethanol immediately after collection. After trimming, the bone samples were dehydrated, infiltrated, and embedded in methyl methacrylate plastic composite [19]. L3 in the sagittal plane were sectioned at 5 and 10 μm using a Reichert–Jung motorized rotary microtome equipped with a tungsten carbide microtome knife. The 5-μm sections were stained with Goldner's trichrome for bright field microscopy and the 10-μm sections were left unstained for epifluorescence microscopy.

Bone histomorphometry was performed using an OsteoMeasure software program version 4.00c (OsteoMetrics, Atlanta, GA, USA) interfaced with a Nikon Eclipse E400 light/epifluorescent microscope and video subsystem. The sampling site of the secondary spongiosa of the vertebral body was performed on an area approximately 1×2 mm within the central portion of the vertebral body, extending dorsoventrally within the marrow space, but excluding the endocortical surfaces. Vigorous validation process was performed prior to the histomorphometry measurement based on Good Laboratory Practices requirements. In particular, only one user performed the measurements with an intra-user precision of 0.72% for BV/TV measurement as an example. The measurements were performed at the middle one third of the lumbar vertebral body and, on average, the marrow cavity of the lumbar vertebral body is about 1.2–1.6×5–6 mm and it seems then hard to fit a region of interest much bigger than 1×2 mm in the cancellous bone of a rat vertebral body. The region of interest may be smaller than usual but the results are quite representative as they are always measured at the same place in each lumbar vertebral body and the sample size (20 to 27) in each treatment group added further assurance. Furthermore, this 2-D histomorphometric measurement method allowed demonstrating a 49.1% OVX-induced bone loss, in accordance with the results found in 3-D histomorphometry, assessed by μCT. We, therefore, consider that this method allowed validating the model of ovariectomy and the following profound deterioration of bone architecture and that the results obtained in strontium ranelate-treated groups can be interpreted. Static parameters (trabecular volume [BV/TV; percent]; trabecular number [Tb.N; number per millimeter]; trabecular thickness [Tb.Th; micrometers]; trabecular spacing [Tb.Sp; micrometers]; osteoid volume [OV/TV; percent]) and dynamic parameters (mineralizing surface [MS/BS; percent]; mineral apposition rate [MAR; micrometers per day]; bone formation rate [BFR/BS; cubic micrometers per square micrometer per year]) were measured in a blinded manner in cancellous bone and were calculated according to the recommendations of the American Society for Bone and Mineral Research committee [20].

Bone nano-indentation

Proximal and distal plateaus of L2 vertebral bodies were cut transversally. The samples were exposed to ultrasound in Deconnex® bath in order to remove the marrow content and rinsed with water. The samples were embedded in polymethyl methacrylate and the faces of the proximal cut were polished and finished with a 0.25-μm diamond spray. The specimens were then hydrated in a 9 g/L NaCl solution for 16 h and nano-indentation tests were performed in wet conditions. A Nano Hardness Tester (CSM Instruments, Peseux, Switzerland) equipped with a three-sided pyramidal Berkovich indenter was used. Five indentations were performed along the center of the posterior cortex of the vertebrae and five indentations in trabecular nodes close to the posterior cortex. The indenter was pressed into the specimens to a 900-nm maximum depth at a rate of 76 mN/min. At maximum load, a 5-s holding period was imposed. The applied load and the penetration depth were continuously recorded during the loading and unloading cycle. Hardness (H) and modulus (E_{it}) were directly calculated from this load–displacement curve using the method previously described [21, 22]. In addition, the dissipated energy that occurred during the deformation of bone was also estimated from this curve.

Bone microtomography

At the end of these evaluations, the embedded L2 vertebral samples were measured by μCT. Parameters of mass and architecture of the secondary spongiosa of the L2 vertebral body were investigated with a high-resolution microcomputer tomography system (μCT 40, Scanco Medical, Bassersdorf, Switzerland). Three-dimensional images of L2 vertebral body were acquired with a voxel size of 20 μm in all spatial directions. The resulting gray-scale

images were segmented using a low-pass filter to remove noise and a fixed threshold to extract the mineralized bone phase. The trabecular and cortical parts of the vertebrae were separated with semi-automatically drawn contours. From the binarized images, structural indices were assessed. Relative bone volume (BV/TV), trabecular number (Tb.N), thickness (Tb.Th), and separation (Tb.Sp) were calculated by measuring directly the 3-D distances in the trabecular network. The mean cortical thickness (Cort.Th.) was also evaluated.

Serum and urine biochemistry

Animals were sedated with a ketamine/xylazine anesthetic for blood sampling from the retro-orbital sinus. Serum samples were obtained on the day of surgery and again during weeks 8, 26, and 52. The effectiveness of ovariectomy was confirmed by radioimmunoassay determination of serum levels of 17beta-estradiol (Diagnostic Products, Los Angeles, CA, USA). To assess bone formation, serum alkaline phosphatase (ALP) was determined colorimetrically on a Roche Cobas Mira auto-analyzer (Roche Diagnostic Systems, Somerville, NJ, USA). In order to assess exposure to circulating strontium ranelate, serum concentrations of strontium were measured at the end of the 52-week treatment period by inductively coupled plasma atomic emission spectrometry (Vista apparatus, Varian).

Urinary samples were collected at week 0 and again during weeks 8, 26, and 52. Prior to urine collections, the animals were placed in metabolic cages and deprived of food for an overnight fast period of 18 h. Centrifuged urine samples were measured for deoxypyridinoline (DPD) by enzyme-linked immunosorbent assay (Quidel, San Diego, CA, USA) to assess bone resorption. The DPD values were normalized to the urinary creatinine, which was determined colorimetrically on a Roche Cobas Mira auto-analyzer (Roche Diagnostic Systems, Somerville, NJ, USA).

Statistical analyses

Results are presented as the means±standard deviations (SD) for all parameters measured and comparisons between groups were done using analysis of variance (ANOVA) [23]. When an overall treatment effect was shown by ANOVA, significant differences vs. the OVX group or the SHAM group were evaluated using Dunnett's multiple comparison procedure or a Fisher test (for μCT and nano-indentation results). For the statistical evaluation of the nano-indentation results, a mean value of five indents was obtained in each bone envelope for each rat and these values were used to calculate the level of significance of the difference between the groups.

Results

Animals

Treatment of OVX rats with 125, 250, or 625 mg/kg/day of strontium ranelate was well-tolerated and safe as it did not have any significant or adverse effects on animal survival, food consumption, body weight changes, clinical signs during the study, terminal body weights, or gross pathology at necropsy (data not shown). Despite pair-feeding, the body weights in all OVX animals (OVX, SR125, SR250, and SR625) slightly increased (from 7.3% to 9.2%) compared to the SHAM rats. However, strontium ranelate had no effect on the body weight gain, confirming that the treatment was well-tolerated. Success of ovariectomy was confirmed at necropsy by the significantly decreased uterine weights and by the lack of detectable levels of serum estradiol in all OVX rats whatever the time point and regardless of treatment, in contrast with SHAM rats exhibiting mean estradiol levels of 8.9±0.5 pg/mL at killing.

Serum strontium concentrations

SHAM and OVX endogenous serum strontium concentrations were similar. After strontium ranelate oral administration for 52 weeks, serum strontium concentration changes were dose-dependent (Table 1). However, the serum strontium concentrations increased 3.4 times between the dose levels of 125 and 625 mg/kg/day, while the given dose increased five times. Therefore, serum strontium concentrations did not increase proportionally to the dose administered, indicating saturation of the strontium absorption processes.

Bone mechanical properties

Compared to SHAM animals, compressive testing of L5 vertebral bodies of OVX animals showed significantly lower

Table 1 Serum strontium concentration (in nanograms per milliliter) obtained at the end of a 52-week treatment with strontium ranelate at 125, 250, and 625 mg/kg/day in OVX rats

SHAM (n=20)	OVX (n=20)	SR125 (n=29)	SR250 (n=26)	SR625 (n=28)
37.8±16.4	55.6±58.4	2,609.9±601.6	4,594.1±1,137.4	8,999.2±2,269.9

Values represent the mean±SD

Table 2 Mechanical strength testing of L5 lumbar vertebra in OVX rats treated with strontium ranelate

	SHAM (n=20)	OVX (n=20)	SR125 (n=27)	SR250 (n=26)	SR625 (n=27)
Maximum load (N)	242.96±59.81** (+46.9)	165.36±51.90 [−31.9]	173.40±51.71 (+4.9)	183.97±45.44 (+11.2)	206.20±45.09* (+24.7)
Stiffness (N/mm)	2,479.30±985.52** (+50.0)	1,653.29±703.75 [−33.3]	1,409.48±433.33 (−14.7)	1,604.18±471.60 (−3.0)	1,651.53±664.46 (−0.1)
Yield load (N)	184.24±54.22** (+46.1)	126.14±31.18 [−31.5]	143.91±41.71 (+14.1)	150.21±36.96 (+19.1)	175.64±35.83** (+39.2)
Energy (mJ)	24.15±17.80* (+78.6)	13.52±6.00 [−44.0]	19.56±9.91 (+44.7)	19.76±11.36 (+46.2)	23.59±10.81** (+74.5)

Values represent the mean±SD. The positive or negative values in parentheses beside the mean values for SHAM and SR groups indicate the percent change vs. OVX. The values in square brackets beside the mean values for the OVX indicate the percent change vs. SHAM
*p<0.05 compared to OVX; **p<0.01 compared to OVX

maximal load (−31.9%, p<0.01, Table 2), stiffness (−33.3%, p<0.01), and energy absorbed (−44.0%, p<0.05). Similarly, the yield load was −31.5% lower (p<0.01). Strontium ranelate-treated OVX rats showed a dose-dependent higher maximal load (up to +24.7%, p<0.05) of the vertebral bodies when compared to OVX controls (Fig. 1a). This was accompanied by a complete prevention of the ovariectomy-induced energy lost in animals treated with 625 mg/kg/day, while stiffness in strontium ranelate-treated animals was unchanged from the values in the OVX animals treated with vehicle (Fig. 1b; Table 2).

Bone mass and architecture

2-D histomorphometry of the L3 lumbar vertebra

Ovariectomy exerted negative effects on cancellous bone structural properties, evidenced by lower BV/TV, Tb.N, and Tb.Th and higher Tb.Sp in OVX vs. SHAM groups for the lumbar vertebra (Figs. 2 and 3). Treatment of OVX rats with 125, 250, or 625 mg/kg/day strontium ranelate prevented the effects of ovariectomy with significant dose-dependent higher BV/TV and Tb.N and lower Tb.Sp

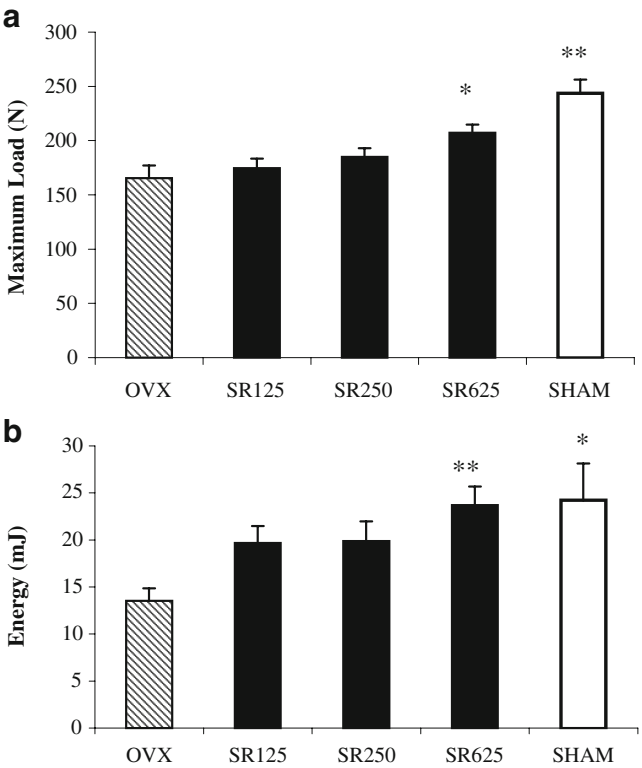

Fig. 1 **a** Maximum load (in newtons) and **b** energy (in millijoules) of L5 lumbar vertebra obtained by a compression test in OVX rats treated with strontium ranelate at 125, 250, and 625 mg/kg/day for 52 weeks. Values represent the mean±SD; n=20–27 animals per group. *p<0.05 compared to OVX control group; **p<0.01 compared to OVX control group

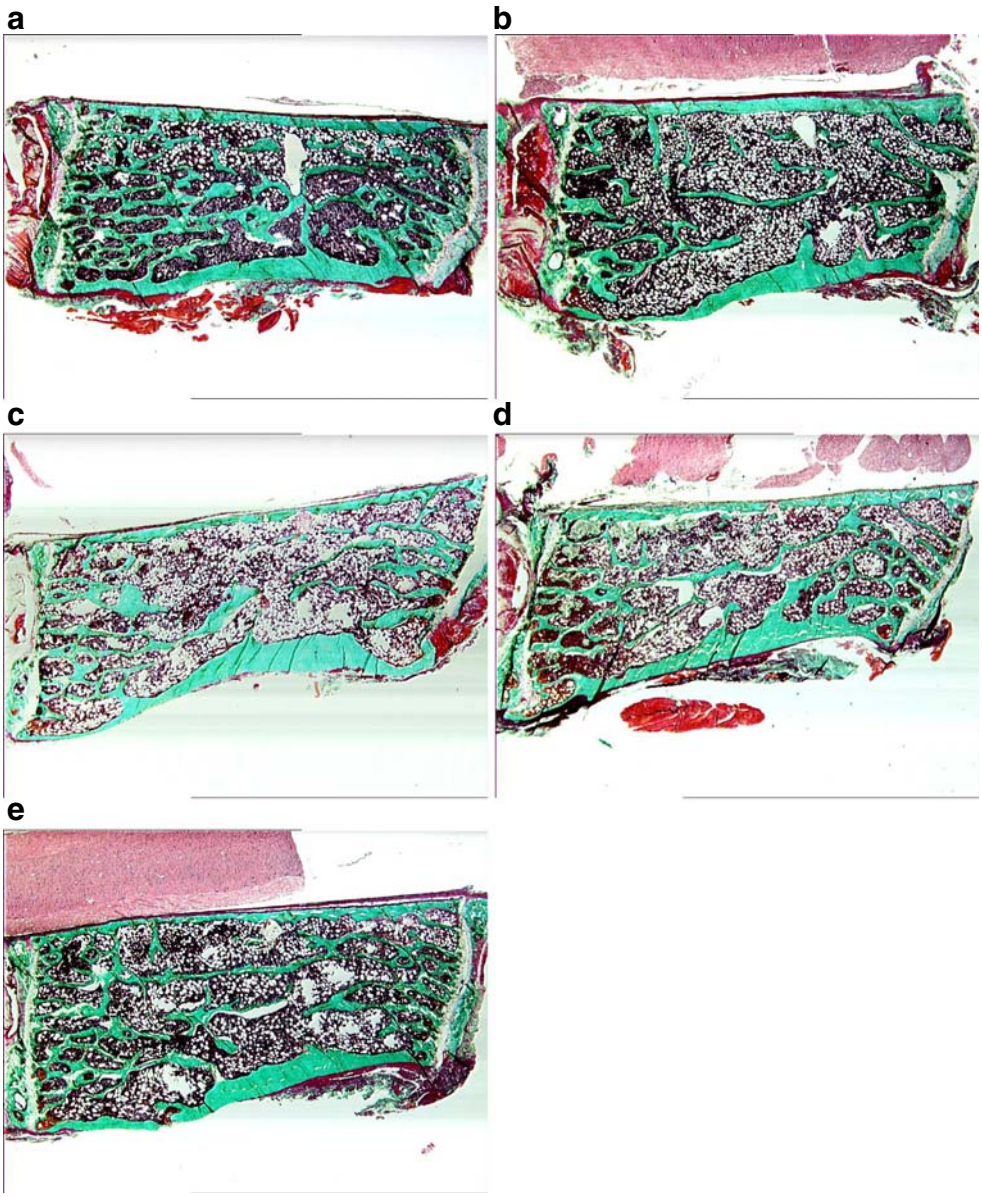

Fig. 2 L3 lumbar vertebra representative pictures of each group (Goldner's trichrome staining, ×10 magnification). **a** SHAM animal; **b** OVX animal; **c**, **d**, and **e** strontium ranelate-treated animals with 125, 250, and 625 mg/kg/day, respectively

compared to OVX control (Figs. 2 and 3). Furthermore, the mean values for Tb.Th in rats treated with 250 mg/kg/day strontium ranelate were as much as +20.2% greater in the lumbar vertebra vs. values in the OVX group, but statistical significance was not achieved.

3-D histomorphometry of the L2 lumbar vertebra

In the OVX control group, BV/TV, Tb.N, and Tb.Th were markedly and significantly decreased (−39.8%, −22.4%, and −7.1%, respectively; Fig. 4). As a consequence, Tb.Sp was significantly increased to +29%. The treatment by strontium ranelate dose-dependently prevented these micro-architecture deteriorations with a significantly higher BV/TV, Tb.N, and Tb.Th than in OVX control animals (up to +36.3%, +12.8%, and +7.6%, respectively, at the highest dose tested, $p<0.05$ to $p<0.001$) and a lower Tb.Sp (up to −12.5% for SR625, $p<0.001$). No statistical changes of the cortical thickness were induced by ovariectomy and by strontium ranelate as well.

Intrinsic bone tissue quality

At the level of the trabecular bone, OVX decreased all the parameters of intrinsic bone tissue quality assessed by the nano-indentation technique (Fig. 5). The differences between OVX and SHAM control groups were statistically significant for hardness (−13.4%, $p<0.05$). In contrast, compared to the OVX control group, all the intrinsic bone tissue quality parameters were significantly higher in rats treated with strontium ranelate whatever the dose level. The highest values were systematically observed in the group receiving SR250, +15.9% for modulus ($p<0.01$), +35.7% for

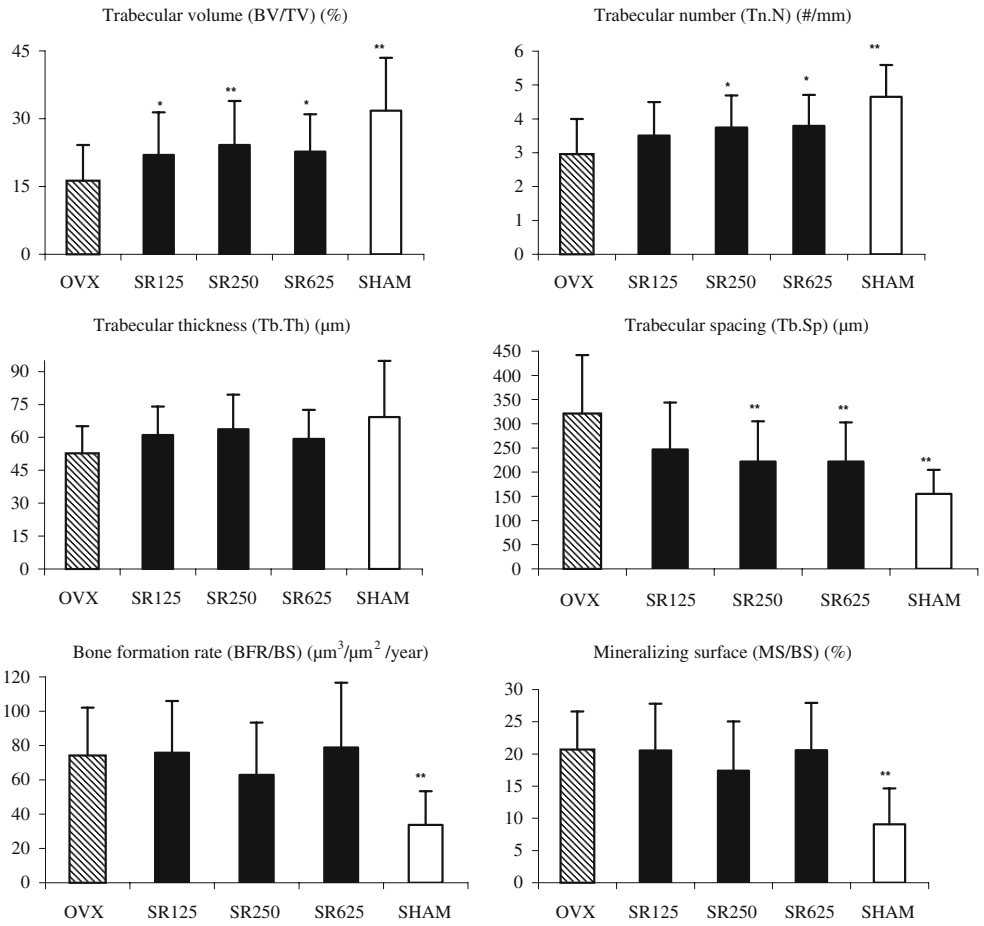

Fig. 3 L3 lumbar vertebra 2-D histomorphometry indices in OVX rats treated with strontium ranelate. Values are expressed as the mean±SD; n=20–27 animals per group. *p<0.05 compared to OVX; ** p<0.01 compared to OVX

hardness (p<0.001), and +18.4% for dissipated energy (p<0.01). Furthermore, in the same strontium ranelate-treated group, SR250, the values were also significantly higher than in SHAM controls for hardness (+17.5%) and dissipated energy (+11.2%). At the level of cortical bone (Fig. 5), no significant differences between the OVX control group and the SHAM control group were observed. However, the hardness was significantly higher in OVX rats treated with strontium ranelate at doses of 125 and 250 mg/kg/day compared to OVX control group (+13.4%, p<0.05, and +17%, p<0.01, respectively).

Bone turnover

Dynamic histomorphometry of the L3 lumbar vertebra

As expected, ovariectomy produced significantly higher dynamic indices of bone formation in the lumbar vertebra (Fig. 3), including bone mineralizing surfaces (MS/BS; +128.8%) and bone formation rate normalized to bone surface (BFR/BS; +121.2%). These changes were consistent with the expected increase in bone formation that accompanies increased bone turnover in estrogen-deficient animals. In rats treated with strontium ranelate, MS/BS and BFR/BS of the lumbar vertebra remained elevated at the high turnover level observed in OVX control rats.

Serum alkaline phosphatase

Treatment of OVX animals with strontium ranelate increased serum levels of ALP, a marker of bone formation, dose-dependently with statistically significant increases of 30.0%, 25.9%, and 23.9% in the SR625 group at the 8-, 26-, and 52-week time points, respectively (p<0.01 compared to OVX; Table 3).

Urinary deoxypyridinoline

Ovariectomy resulted in increased mean levels of urinary DPD, a biomarker of bone resorption at the 8-, 26-, and 52-week time points (Table 3) with a maximal effect 8 weeks after ovariectomy. In addition, there was a notable age-related decline in this parameter that appeared to reach a steady state by the 26-week time point (i.e., around the age of 1-year-old). Strontium ranelate treatment reduced the DPD levels by 20.4%, 13.9%, and 21.1% (p<0.05 compared to OVX for SR625, Fig. 1b) in the SR125, SR250, and SR625 groups, respectively, at the 8-week time point.

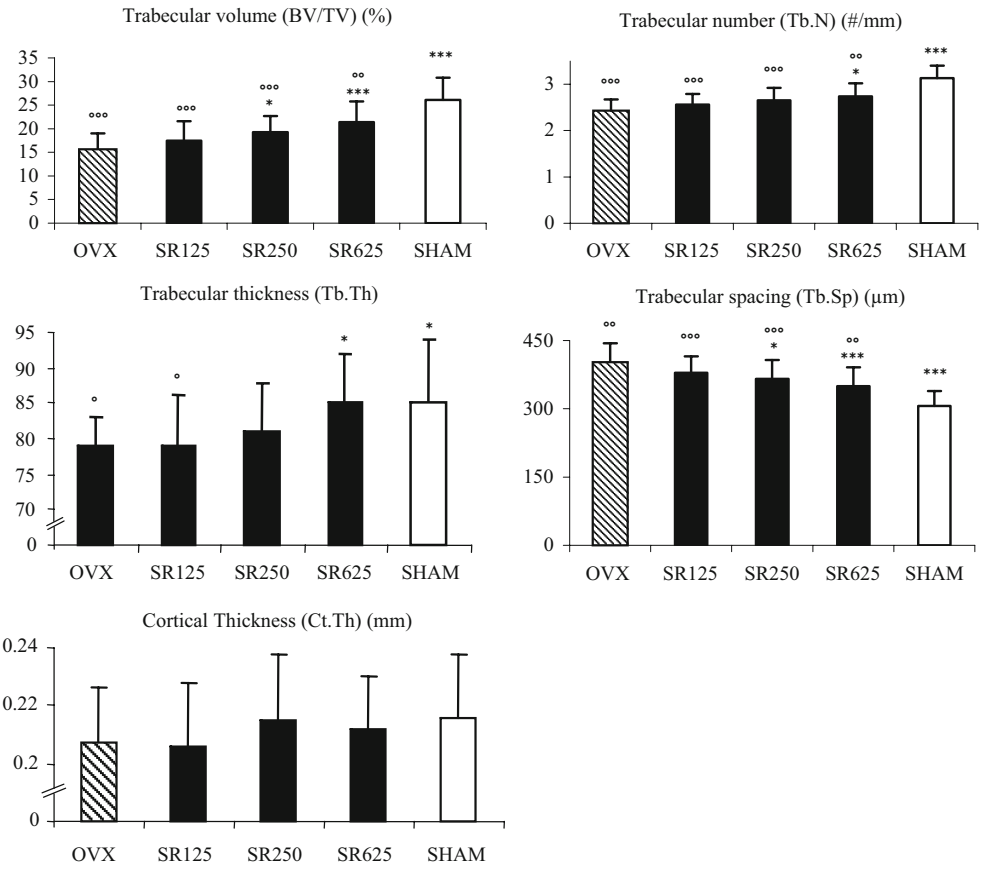

Fig. 4 L2 lumbar vertebra 3-D histomorphometry indices assessed by µCT in OVX rats treated with strontium ranelate. Values represent the mean±SD; $n=12$ animals per group. *$p<0.05$ compared to OVX; **$p<0.01$ compared to OVX; ***$p<0.001$ compared to OVX. °$p<0.05$ compared to SHAM; °°$p<0.01$ compared to SHAM; °°°$p<0.001$ compared to SHAM

Discussion

The results indicate that ovariectomy decreased lumbar vertebra bone strength by altering bone mass, bone microarchitecture, and intrinsic bone tissue quality, which is consistent with previously published findings observed in this model [16–18, 24–30]. In contrast, in the conditions of this study, there were no reductions in the mechanical strength of the midshaft femur in OVX control rats 52 weeks after ovariectomy when compared to SHAM control rats (mean maximum load±SD=182.64±17.85 N for OVX vs. 171.41±17.47 N for SHAM). This may be explained by the lack of OVX-induced cortical bone loss (no change of cortical width, data not shown) and by an increase in cross-sectional geometries between SHAM and OVX animals (data not shown), as previously described in this model [31, 32]. The combination of these two effects is consistent with the lack of differences in long bone strength between the SHAM and OVX control groups as the resistance to bending loads is primarily governed by the midshaft's geometric configuration. Therefore, the absence of effects of ovariectomy on long bones midshaft cortical strength preclude any interpretation in our experimental conditions.

Compared to vehicle-treated OVX controls, the treatment of OVX rats with strontium ranelate for 1 year at doses of 125, 250, and 625 mg/kg/day was well-tolerated and safe. These dose levels lead to mean serum strontium concentrations which correspond, respectively, to 0.25-, 0.44-, and 0.87-fold the median serum strontium concentrations observed in patients administered the therapeutic dose of 2 g/day (i.e., 10,560 ng/mL, [4]) and receiving 1,500 mg calcium/day. As illustrated in the present study, serum strontium concentrations did not increase proportionally to the dose administered (serum strontium concentrations increased of only 3.4 times when dose levels increased of five times), indicating a saturation of the strontium ranelate absorption processes when doses increase. As the same transport properties that have been developped and extensively used for studies of calcium absorption also apply to strontium [33], it is assumed that some portions of strontium are absorbed into blood from the intestinal lumen via passive nonsaturable diffusion while another portion proceeds by an active saturable transport [34] which can explain why calcium and strontium can compete during their absorption process. Interestingly, this is illustrated in a recent publication [35] where OVX rats receiving 150 mg/kg/day of strontium

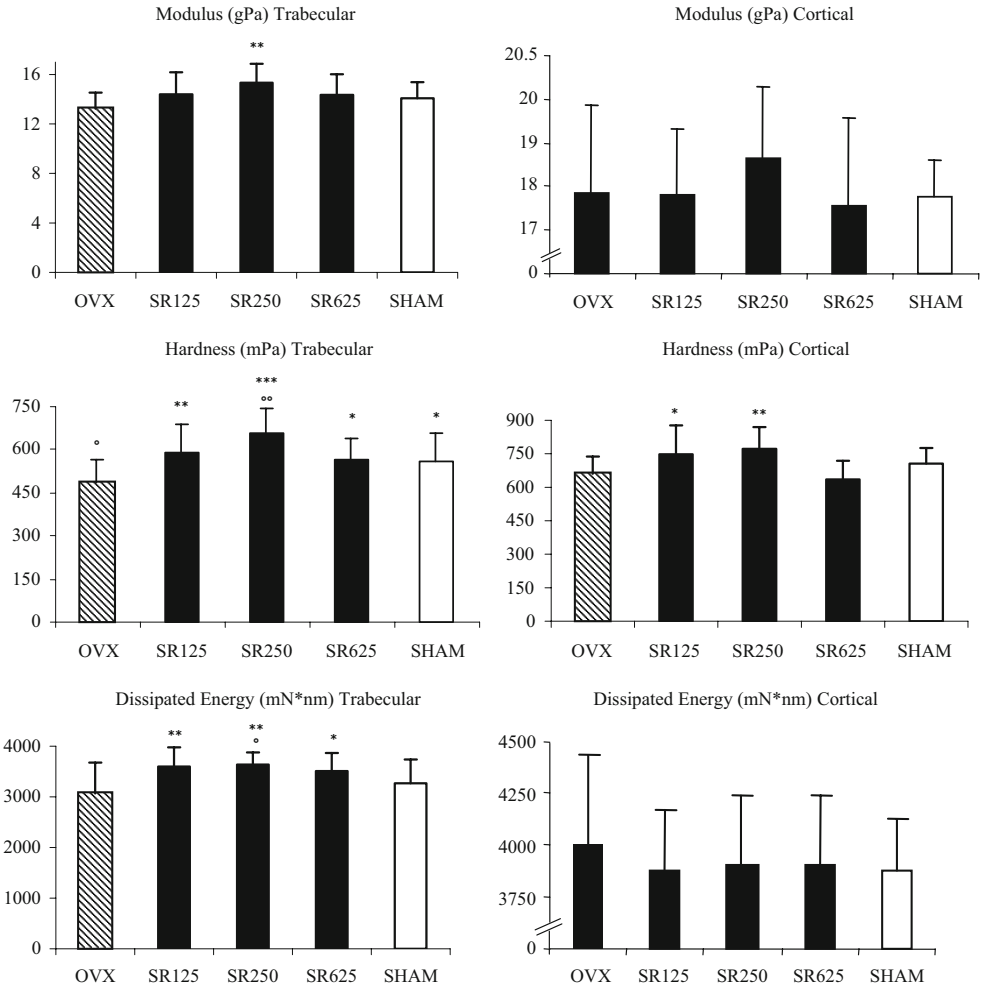

Fig. 5 Intrinsic trabecular and cortical bone quality testing of L2 lumbar vertebra in OVX rats treated with strontium ranelate. Values represent the mean±SD; $n=12$ animals per group. *$p<0.05$ compared to OVX; **$p<0.01$ compared to OVX; ***$p<0.001$ compared to OVX. °$p<0.05$ compared to SHAM; °°$p<0.01$ compared to SHAM

ranelate with a normal calcium diet (1.19% Ca) exhibited, as expected, a sevenfold less serum strontium concentration than the same dose given with a calcium-deficient diet (0.1% Ca) due to the competition in the absorption process of both cations. In the Fuchs et al. paper [35], when normal calcium diet was given to OVX rats, the serum strontium concentration obtained at 150 mg/kg/day was comparable to the one observed in the present study at the dose of 125 mg/kg/day. Nevertheless, these concentrations represent a fourfold lower strontium concentration than the one observed in treated patients at the therapeutic dose which explains their lack of efficacy. It is also interesting to note that, in this recent publication [35], strontium ranelate at 150 mg/kg/day (but administered with a calcium-deficient diet) exhibited no efficacy in OVX rats while leading to a higher serum strontium concentration than the efficient dose of 625 mg/kg/day in the present paper (administered with normal calcium diet). This confirms that no beneficial effect on bone is possible if the amount of calcium available from the diet is not sufficient. It is indeed described in the literature that ovariectomy worsens the calcium deficiency-induced hyperparathyroidism leading to decreases in bone calcium content, bone mineral density, and bone strength [36, 37]. Therefore, as recommended for all drugs used for osteoporosis treatment, a normal calcium intake is necessary and only serum strontium concentrations obtained in OVX rats fed a normal calcium diet are relevant for human therapeutic interpretation.

In the present study, at the dose of 625 mg/kg/day, leading in OVX rats fed a normal calcium diet to serum strontium concentration close to those observed in treated patients, the maximum load and total energy absorbed by the vertebra before fracture were equivalent to SHAM animals, indicating a complete prevention of the ovariectomy-induced loss of bone strength.

In the strontium ranelate-treated OVX rats, BV/TV values were intermediate between those of OVX and SHAM control groups, as indicated by the significant difference between the BV/TV measured by 2-D and 3-D histomorphometry and the OVX and SHAM controls. The prevention in BV/TV values degradation was accompanied by corresponding dose-dependent positive effects of strontium ranelate on bone micro-architecture as evidenced by higher Tb.N and Tb.Th. Importantly, the two different methods used

Table 3 Bone formation (ALP) and bone resorption markers (urinary DPD) in OVX rats treated with strontium ranelate

		SHAM (n=20–22)	OVX (n=22–25)	SR125 (n=28–30)	SR250 (n=27–30)	SR625 (n=27–30)
ALP (IU/L)	Baseline	162±22 (+1.3)	160±33 [−1.2]	159±37 (−0.6)	161±23 (+0.6)	161±42 (+0.6)
	Week 8	159±26 (−0.6)	160±36 [+0.6]	156±34 (−2.5)	178±34 (+11.3)	208±35** (+30.0)
	Week 26	153±24 (−3.2)	158±28 [+3.3]	156±28 (−1.3)	161±30 (+1.9)	199±47** (+25.9)
	Week 52	170±33 (−5.6)	180±53 [+5.9]	182±53 (+1.1)	193±39 (+7.2)	223±48** (+23.9)
DPD (nM/mM creatinine)	Baseline	27.7±9.9 (−17.6)	33.6±17.3 [+21.3]	36.9±33.2 (+9.8)	31.9±12.0 (−5.1)	37.0±17.6 (+10.1)
	Week 8	27.5±14.9** (−74.0)	105.7±37.8 [+284.4]	84.1±27.5 (−20.4)	91.0±34.2 (−13.9)	83.4±30.4* (−21.1)
	Week 26	15.4±5.4** (−49.3)	30.4±11.8 [+97.4]	30.3±8.8 (−0.3)	33.5±14.4 (+10.2)	31.5±7.1 (+3.6)
	Week 52	13.6±5.0** (−66.3)	40.4±18.1 [+197.1]	38.1±15.0 (−5.7)	37.3±18.0 (−7.7)	43.1±19.3 (+6.7)

Values represent the mean±SD. The positive or negative values in parentheses beside the mean values for SHAM and SR groups indicate the percent change vs. OVX. The values in square brackets beside the mean values for the OVX indicate the percent change vs. SHAM
*p<0.05 compared to OVX; **p<0.01 compared to OVX

to assess these parameters, namely, 2-D and 3-D histomorphometry, led to the same results, suggesting a negligible influence of strontium on bone μCT assessment in these conditions. It is important to note that bone strength was assessed at the level of the whole vertebral body; the measured bone strength reflects both the contribution of the trabecular and cortical compartments on bone resistance. In contrast, micro-architecture was assessed, whatever the method used, only at the trabecular level which can explain why the partial effect on micro-architecture cannot totally explain the full rescue of the biomechanical effect on bone strength.

Evaluation of the bone biomarkers and dynamic indices of bone formation provide further insight regarding strontium ranelate's beneficial action on bone. Interestingly, the effects on bone resorption markers of strontium ranelate after ovariectomy combined a modest decrease in urinary DPD (observed mainly at 625 mg/kg/day and at the 8-week time point when ovariectomy induced the maximal increased level of bone resorption), in line with a decreased osteoclastic bone resorption [12, 13, 38] observed together with a dose-dependent increase in the serum levels of ALP in OVX animals, consistent with previous data [9]. Taken together, these observations indicate that the ability of strontium ranelate to prevent the ovariectomy-induced bone loss is due to a combination of its inhibitory effects on bone resorption and its ability to stimulate and/or maintain high levels of bone formation. Maintaining elevated indices of bone formation in OVX animals provides evidence for an effect on bone formation [9, 14, 38] and the dynamic indices of bone formation such as mineralizing surface and bone formation rate are coherent with the bone markers changes observed. However, cellular parameters (such as osteoclast and osteoblast number and surface) were not measured in this study because their changes, 52 weeks after ovariectomy when the adult rats were more than 18 months old, were anticipated to be undetectable compared to SHAM controls. It is indeed described in Wronski et al. [26] that, from 150 days after ovariectomy, osteoblast surfaces and osteoclast surfaces in OVX rats declined to SHAM control levels. Furthermore, osteoblasts are difficult to identify in old animals and this can result in a high variability of the cellular parameters. Static cellular parameters are not indicative of cellular activity and corresponding bone formation and resorption rates. They can only give information at a given time, without allowing an extrapolation for the whole treatment period. In contrast, dynamic fluorochrome-based parameters are meaningful as they provide strong evidence for a high bone formation rate. In OVX strontium ranelate-treated rats, fluorochrome-based indices of bone formation were maintained high while bone mass decrease was prevented, indicating that the level of bone formation exceeds the level of bone resorption.

In younger OVX rats [9], strontium ranelate decreased histomorphometric indices of bone resorption to the levels in SHAM animals; in contrast to this inhibitory effect on bone resorption, osteoblast surfaces were as high in OVX rats treated with strontium ranelate as those in control OVX rats treated with vehicle. The positive strontium ranelate effect on bone mass and bone micro-architecture can then be related to the cellular effect observed in previous in vitro and in vivo studies which indicate that strontium ranelate acts through a dual mechanism of action [38], namely, reduction of osteoclastic bone resorption [12, 13] and stimulation of osteoblastic bone formation [14].

The contribution of intrinsic bone tissue quality, using nano-indentation tests in wet conditions, was assessed at the level of the dorsal cortex of the vertebral body and of the trabecular nodes close to this region. Previous studies indicated that this latter area of the vertebral body is markedly affected by low protein regimen or treatments of osteoporosis [21]. The results of the present study confirm

that trabecular bone intrinsic quality is affected by OVX and that the treatment with strontium ranelate at each investigated dose resulted in a significant higher hardness and dissipated energy in this type of bone. Whatever the strontium ranelate dose, these values were higher than in SHAM controls. These results indicate that the load and energy required to induce a given deformation of a bone lamella are markedly increased by strontium ranelate treatment in OVX rats. The bone tissue formed under strontium ranelate treatment shows improved intrinsic quality properties, suggesting that it is able to withstand greater damage before fracture. The fact that cortical bone is poorly affected by OVX is not surprising, since in rodents there is little remodeling of the cortical bone and since its vascularization is poorly developed. Furthermore, the treatment started in mature rats in which no periosteal apposition could be observed. However, even in these conditions, strontium ranelate was able to increase hardness at two dose levels. This is in line with a previous study performed in intact rats treated during all their life with strontium ranelate where a mild but significant effect of the treatment was observed at the level of the cortical bone [39].

How strontium ranelate totally prevented and even improved the OVX-induced deterioration of intrinsic bone tissue quality is still under investigation. Indeed, strontium ranelate does not affect crystal properties and characteristics as shown in vivo [40, 41]: the normal mineralization process and the mean degree of mineralization of bone are preserved, and when strontium takes the place of calcium, only one calcium ion out of ten is substituted by one strontium ion in the hydroxyapatite crystal lattice. The majority of strontium present in bone is adsorbed onto the surface of the crystal, in the nonapatitic hydrated layer [42]. This leads to the hypothesis that this strontium localization in the bone tissue could potentially lead to a better cohesion between the mineral and the protein matrix and/or to a direct or indirect effect (through the cellular activity) on the spatial orientation of the crystals in the lamellae or between the lamellae themselves.

In conclusion, strontium ranelate effects on bone mass, on trabecular micro-architecture, and on the intrinsic properties of the material allow explaining, at the dose level of 625 mg/kg/day, the prevention of OVX-induced biomechanical deterioration. Long-term strontium ranelate treatment prevents the OVX-induced deterioration of bone mechanical properties acting on all their main determinants in adult rats at a therapeutic equivalent human dose level. This therapeutic effect was associated with a bone formation rate maintained at a high level together with a slight decrease of bone resorption. Taken together, observations reported in this study support the efficacy and the safety of strontium ranelate and contribute to explain strontium ranelate antifracture efficacy for the treatment of postmenopausal osteoporosis.

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Conflicts of interest None.

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